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Chemical characterization of water and ethanolic extracts of Turkish propolis by HPLC-DAD and GC-MS

Tuğba Nigar Bozkuş^a , Orhan Değer^b , and Ahmet Yaşar^c 

^aLaboratory Technology Program, Artvin Vocational School, Artvin Coruh University, Artvin, Turkey; ^bDepartment of Medical Biochemistry, Faculty of Medicine, Karadeniz Technical University, Trabzon, Turkey; ^cDepartment of Basic Pharmaceutical Sciences-Analytical Chemistry, Faculty of Pharmacy, Karadeniz Technical University, Trabzon, Turkey

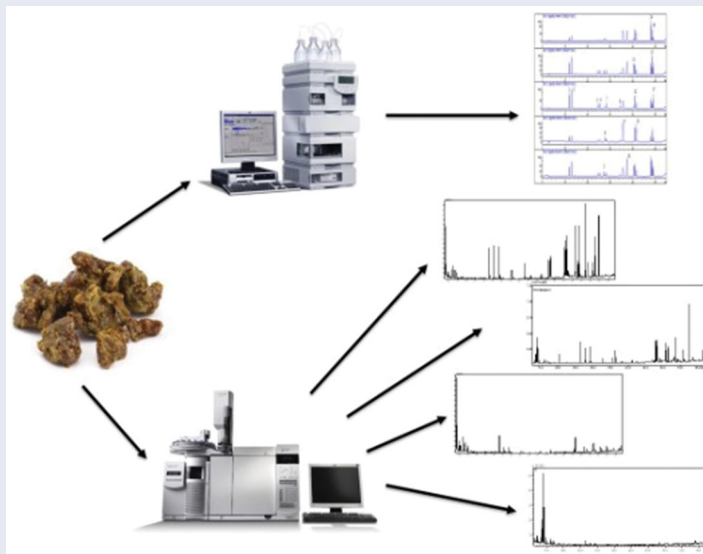
ABSTRACT

This study aims to determine the qualitative and quantitative contents of Turkish propolis collected from various regions of Turkey using high-performance liquid chromatography with the diode-array detector (HPLC-DAD) and gas chromatography-mass spectrometry (GC-MS) in water and ethanolic extracts. In HPLC-DAD analyses, it was determined that water extract of Turkish propolis contains phenolic acids such as caffeic acid (204.00 µg/mL), trans-cinnamic, chlorogenic, and caffeoylquinic acids, responsible for its antioxidant activity; whereas, the ethanolic extract of Turkish propolis contains chrysin (641.33 µg/mL), caffeic acid phenethyl ester (630.67 µg/mL), pinocembrin (572.67 µg/mL), galangin (534.11 µg/mL), naringenin (372.39 µg/mL), and also kaempferol, trans-cinnamic acid, caffeic acid, myricetin, and quercetin. GC-MS analyses showed that ethanolic extract of propolis contains caffeic acid by Rtx-1 column and the water extract of propolis contains quinic acid and ferulic acid by Rtx-5ms column. Various sugar derivatives were detected by both columns in water and ethanolic extracts of Turkish propolis. HPLC-DAD can be considered as a more effective method than GC-MS for the chemical characterization of propolis. Water extract of Turkish propolis can be a good source of raw materials for various sectors, as it is both cheap and has less health risk than ethanolic extract, and is suitable for human use.

KEYWORDS

Ethanolic extract of propolis; GC-MS; HPLC-DAD; polyphenols; water extract of propolis

GRAPHICAL ABSTRACT



Introduction

Propolis (bee glue) is a sticky, natural substance collected by honeybees (*Apis mellifera*) from various plant sources using for closing holes of their hives, smoothing internal walls, protecting entry against the wind, rain, and outside invader such as snake, lizard.^[1,2] Propolis has different colors such

as dark yellow, green, red, and brown, depending on its geographic region, plant source, age and it has a characteristic odor due to volatile oils of its contents.^[3]

Generally, propolis consists of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen, and 5% various substances containing other remains.^[4] Propolis contains various chemical components such as

polyphenols (flavonoid aglycones, phenolic acids, and their esters, phenolic aldehydes, alcohols, and ketones), sesquiterpene quinones, coumarins, steroids, amino acids, and organic compounds.^[5]

The presence of a large number of flavonoids, aromatic acids, and phenolic compounds has been suggested to be responsible for many biological and pharmacological activities of propolis.^[6] As propolis cannot be used as raw material, it must be purified with solvent extraction.^[7] Although ethanolic extract of propolis is commonly used, concerning the study of water extract of propolis is on the increase. Up to the present, water extract of propolis has been reported to exhibit hepatoprotective activity, antiviral activity, inhibition of platelet aggregation, and anti-inflammatory activity in both chemical and immunological liver injury models and have good antioxidant activity with a high content of polyphenolic compounds.^[8] Most of the substances in propolis are lipophilic compounds. Since it is particularly easy to extract lipophilic compounds using ethanol and also particularly suitable for obtaining a propolis extract rich in polyphenolic compounds from which has been removed from resin, ethanolic extract of propolis is well known.^[7,9] It has been reported that the water extract of propolis and its major constituents containing caffeoylquinic acids have higher antioxidant activity and high inhibitor/activator effect against certain enzymes.^[9]

Chromatographic techniques such as gas chromatography and especially HPLC, provide the profile and identification of each polyphenolic compound.^[10] Just as detection by HPLC is often a powerful tool for the detection of compounds based on the measurement of UV absorption, often using DAD, GC-MS is an excellent technic to detect volatile substances.^[11,12]

Different solvents used to extract the propolis will dissolve the different compounds in the sample. Therefore, this study aimed to prepare water and ethanolic extracts of Turkish propolis collected from various regions of Turkey and to determine the content qualitatively and quantitatively using HPLC-DAD and GC-MS techniques. It is also aimed to analyze the water extract of propolis prepared by our method in our laboratory and to compare it with the ethanolic extract to bring up the usability as a food supplement for humans due to in particular its strong antioxidant content. In this study, the characterization of the water extract of Turkish propolis is studied for the first time. In previous studies, the content of the ethanolic extract of Turkish propolis was determined only by GC-MS. In addition to GC-MS analyses, the content, in particular, polyphenols, obtained by HPLC-DAD is also included in this study.

Materials and methods

Chemicals and reagents

Caffeic acid phenethyl ester ($\geq 97\%$), *p*-coumaric acid ($\geq 98\%$), naringin ($\geq 95\%$), apigenin ($\geq 95\%$), kaempferol ($\geq 97\%$), myricetin ($\geq 96\%$), galangin (autophagy including flavonoid), quercetin ($\geq 95\%$), 3,4-di-O-caffeoylquinic acid ($\geq 90\%$), 4,5-di-O-caffeoylquinic acid ($\geq 85\%$) and

acetonitrile ($\geq 99\%$) were purchased from Sigma. Caffeic acid ($\geq 98\%$), naringenin ($\geq 95\%$), chlorogenic acid ($\geq 95\%$), trans-cinnamic acid ($\geq 99\%$) were obtained from Aldrich. Ethanol ($\geq 99.8\%$), acetic acid ($\geq 99.7\%$), pyridine ($\geq 99.9\%$) were purchased from Sigma-Aldrich (St. Louis, USA). Pinocembrin (95%) and N,O-bis (trimethylsilyl) trimethylchlorosilane with trifluoroacetamide (1 mL, including 1% TMCS, 99% (except TMCS)) were obtained from Fluka. 3,4,5-tri-O-caffeoylquinic acid ($> 98\%$) was supplied by Chengdu Biopurify Phytochemicals Ltd. (Mallinckrodt, Mexico), and 3,5-di-O-caffeoylquinic acid ($\geq 85\%$) was purchased from Santa Cruz Biotechnology (Shanghai, China). All solvents were analytical grade. Analytical grade solvents were used for HPLC and GC-MS determinations. Ultrapure water was purified by Pure Lab Classic from Elga Lab Water (Elga, UK) was used through this work.

Sample collection

Propolis samples were collected from four different regions of Turkey from Fanus Nutrient Commerce Anonymous Company (Trabzon) and kept in the freezer (-20°C) until further use. The locations of samples from Turkey were Trabzon (North of Turkey), Erzurum (East of Turkey), Zonguldak (West of Turkey), and Adıyaman (South of Turkey). These four different cities of Turkey to the north, south, east, and west were selected since they represent the four separate geographical locations.

Preparation of water and ethanolic extracts of Turkish propolis samples

Natural propolis samples collected from various regions of Turkey and frozen at -20°C were grated and re-frozen at -20°C . Grated propolis samples were pulverized in a blender and the powders were combined into a mixture. In this way, a mixed propolis sample called Turkish propolis was obtained. Propolis samples (0.5 g) were extracted with deionized water (20 mL) and absolute ethanol (20 mL) at 60°C by shaking at 150 rpm for 24 hours. Then each extract was centrifuged at 4000 rpm (2057 g) for 10 min at 4°C . The extracts were filtered through a filter paper and kept refrigerated in the dark at 4°C before analyses. The final concentrations of the extracts were 25 mg/mL. Extraction yields (%) were calculated by proportioning the dry propolis masses before and after extraction and multiplying by 100. The yield of water and ethanol extractions were 20% (w/w) and 90% (w/w), respectively.

Preparation of standard solutions

Caffeic acid, caffeic acid phenethyl ester, apigenin, myricetin, kaempferol, quercetin, galangin, chrysin, naringin, trans-cinnamic acid, pinocembrin, and naringenin stock solutions were prepared in 100% methanol, 3,4,5-tri-O-caffeoylquinic acid stock solution was prepared in 50% methanol, chlorogenic acid stock solution was prepared in 40% methanol, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid,

4,5-di-O-caffeoylquinic acid stock solutions were prepared in deionized water. All stock standard solutions were prepared as 1 mg/mL and filtered through 0.2 µm disposable syringe filters, then stored at 4 °C in the dark. The working solutions were obtained by diluting the stock standard solutions of the 17 phenolic compounds (10 µg/mL) with 40% methanol. They were mixed to prepare standard mixture solution (std mix) and they were diluted to a series of working standard solutions with different concentrations (50, 25, 10, 5, and 2.5 µg/mL) for further working curve construction.

The whole standards were prepared and analyzed three times. The concentrations of standards were determined based on the slope of the standard curves. All standard concentrations were determined using linear calibration curves based on the peak area for each standard. The calibration curves were in the range of 2.5–50 µg/mL of polyphenol standards.

HPLC-DAD analyses

Chromatographic conditions

The propolis extracts were analyzed by HPLC Agilent 1100 Series (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a vacuum degasser, quaternary pump, auto-sampler, column thermostat, multi-wave UV/VIS detector, and diode array detector (DAD). Chromatographic separations were performed using a Thermo Scientific ODS-2 Hypersil column (250 × 4.6 mm, 5 µm particle size) with a Symmetry C18 guard column (3.9 mm i.d. × 20 mm length). The mobile phase comprised of 2% acetic acid in water (A), 0.5% acetic acid in water:acetonitrile (1:1) (B) and acetonitrile (C) using gradient elution programme: 0 min (5% B); 5 min (5% B); 8 min (20% B); 10 min (22% B); 17 min (25% B); 19 min (27% B); 30 min (40% B); 35 min (45% B); 40 min (65% B); 43 min (70% B, 1% C); 45 min (80% B, 2% C); 48 min (90% B, 4% C); 50 min (100% B); 52 min (100% C); 53 min (5% B); 55 min (5% B). The column temperature was kept at 25 °C and the injection volume was set at 20 µL. The flow rate was maintained at 1.2 mL/min and the detection wavelengths were set at 265 nm (for chrysin and galangin), 280 nm (for naringin and trans-cinnamic acid), 290 nm (for naringenin and pinocembrin), 320 nm (for chlorogenic acid, caffeic acid, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid, 3,4,5-tri-O-caffeoylquinic acid, apigenin, and caffeic acid phenethyl ester) and 360 nm (for myricetin, quercetin, and kaempferol). The calibration curve for each standard was established among the concentration range of 2.5–50 µg/mL. The calibration curves were obtained by plotting the peak area of the compound at each level against the concentration of the sample. The correlation coefficients are presented in Table 1.

Validation of the method

To further validate and evaluate the performance of the analytical HPLC method established, limit of detection (LOD)

Table 1. Retention time, regression equation, correlation coefficient, percent relative standard deviation, LOD, and LOQ results obtained from calibration curves of polyphenol and caffeoylquinic acid standards.

Compound No	Compound name	Retention time (RT) (min)	Regression equation ^a	Correlation coefficient (r ²)	% RSD (area)	LOD (µg/mL)	LOQ (µg/mL)
1	Chlorogenic acid	11.813	Y = 45.896X – 4.66	0.9967	1.458	0.373	1.245
2	Caffeic acid	13.067	Y = 87.661X – 18.527	0.9952	1.829	0.497	1.655
3	4,5-di-O-caffeoylquinic acid	24.662	Y = 25.111X – 12.05	1	2.947	0.884	2.948
4	3,5-di-O-caffeoylquinic acid	25.340	Y = 49.586X – 9.55	1	1.078	0.324	1.079
5	Naringin	27.272	Y = 24.91X + 0.637	0.9979	1.673	0.448	1.492
6	Myricetin	27.845	Y = 54.016X – 99.099	0.9996	1.206	0.386	1.286
7	3,4-di-O-caffeoylquinic acid	28.424	Y = 38.169X – 25.85	0.9929	1.739	0.528	1.760
8	3,4,5-tri-O-caffeoylquinic acid	34.422	Y = 47.26X – 53.614	0.9996	2.995	0.892	2.973
9	Quercetin	35.670	Y = 60.978X – 185.38	0.9953	1.093	0.358	1.192
10	Trans-cinnamic acid	37.439	Y = 130.25X + 26.669	0.9986	1.264	0.336	1.118
11	Naringenin	40.520	Y = 58.406X – 20.871	0.9997	0.600	0.171	0.570
12	Apigenin	40.953	Y = 66.369X – 23.919	0.9981	0.648	0.191	0.636
13	Kaempferol	41.611	Y = 62.683X – 41.648	0.9994	1.365	0.381	1.269
14	Chrysin	47.974	Y = 93.669X – 61.048	0.9965	2.816	0.757	2.524
15	Pinocembrin	48.447	Y = 73.832X – 60.166	0.9991	0.072	0.072	0.239
16	Galangin	48.744	Y = 67.682X – 69.854	0.9984	4.053	1.116	3.721
17	Caffeic acid phenethyl ester	49.145	Y = 59.521 – 23.245	0.9994	2.729	0.769	2.563

^aX is the concentration of compounds; Y is peak area; RSD: relative standard deviation; LOD: limit of detection; LOQ: limit of quantification.

and limit of quantification (LOQ) for each compound were defined as 3 and 10 times the standard deviation (σ) at the triple concentrations predicted for a real sample. LOD and LOQ were calculated and the detailed results are shown in Table 1.

GC-MS analyses

Derivatization

25 mg/mL ethanolic and water extract of Turkish propolis were filtered through 0.45 μ m disposable syringe filters. The ethanolic extract of Turkish propolis was evaporated under a vacuum below at 50 °C. The water extract of Turkish propolis was frozen at –80 °C and dried with a lyophilisator. Each 7 mg sample of the dried extract was mixed with 350 μ L of pyridine and 700 μ L of (bis-trimethylsilyl) trifluoroacetamide (BSTFA) including 1% trimethylchlorosilane (TMCS) in a glass vial, heated at 100 °C for 30 min to prepare the samples before their injection (2 μ L) into the GC-MS.

GC-MS analyses with Rtx-1 and Rtx-5ms columns

A Shimadzu Model GC-2010 Series gas chromatograph, coupled with a Shimadzu series mass-selective detector quadrupole mass spectrometer model GCMS-QP 2010 plus and flame ionization detector (70 eV ionization voltage), was used (Shimadzu, Kyoto, Japan). The GC-MS system is equipped with Rtx-1 and Rtx-5ms (Restek) capillary columns (30 m \times 0.25 mm i.d., 0.25 μ m film thickness). Helium was used as carrier gas at a flow rate of 1 mL/min. The ionization voltage was 70 eV. Initially, the temperature was held at 60 °C for 2 min, then it was raised to 240 °C at a rate of 3 °C/min, and maintained at 240 °C for 62 min. The samples (2 μ L) were injected in the split mode at 250 °C. The peaks were defined by computer searches of the National Institute of Standards and Technology (NIST) and Wiley commercial reference libraries.^[6] Identification of the chemical compounds of propolis by GC-MS was based on the peak area and retention time.

Results

HPLC-DAD analyses

Chemical composition of water and ethanolic extract of Turkish propolis by HPLC-DAD

HPLC-DAD chromatograms of standard polyphenol and caffeoylquinic acid compounds are shown in Figure 1. Table 2 shows the qualitative and quantitative analysis of identified compounds in water and ethanolic extracts of Turkish propolis. The amount of the polyphenols and caffeoylquinic acids are given as μ g polyphenol or caffeoylquinic acid/mL water or ethanolic extract of propolis. HPLC-DAD analysis revealed that the presence of two phenolic and two caffeoylquinic acid compounds in the water extract of Turkish propolis and ten phenolic compounds (seven flavonoids, two phenolic acids, and one phenolic acid ester) in the ethanolic extract of Turkish propolis. The most abundant constituent of water extract of Turkish propolis was caffeic acid (204.00 μ g/mL). In

the ethanolic extract of Turkish propolis, the most abundant constituent was chrysin (641.33 μ g/mL). Caffeic and trans-cinnamic acids were detected as common compounds in both extracts. In the water extract of Turkish propolis, caffeic acid was determined at a much higher rate than the ethanolic extract, while trans-cinnamic acid was found to be higher in the ethanolic extract than the water extract.

Method validation

As shown in Table 1, LOD values were in the range 0.072–1.116 μ g/mL for polyphenols and 0.324–0.892 μ g/mL for caffeoylquinic acids. LOQ values were in the range of 0.239–3.721 μ g/mL for polyphenols and 1.079–2.973 μ g/mL for caffeoylquinic acids. These results demonstrate that the proposed HPLC-DAD method is sufficiently sensitive for the determination of polyphenols and caffeoylquinic acids in propolis samples.

GC-MS analyses

Chemical composition of water and ethanolic extract of Turkish propolis by GC-MS with Rtx-1 and Rtx-5ms columns

Chemical composition of water and ethanolic extract of Turkish propolis were analyzed by gas chromatography-mass spectrometry (GC-MS) and the main compounds and their percentages of area are given in Tables 3–6. The GC-MS analysis of the propolis samples identified 21 and 37 compounds in the water extracts of propolis using Rtx-1 (for the detection of non-polar components) and Rtx-5ms column (for the detection of low polarity components) (Tables 3–4), respectively. Similarly, 24 and 14 substances, respectively, were determined in the ethanolic extracts of propolis using these columns (Tables 5 and 6). The analysis using Rtx-5ms column revealed that the water extract of propolis contained quinic acid (0.23%), ferulic acid (0.27%), cinnamic acid derivatives, and proline amino acid. In the analysis using Rtx-1 column, the ethanolic extract of propolis contained caffeic acid (1.91%) and cinnamic acid derivatives. Benzoic acid was found in all of the analyses of both water and ethanolic extract of propolis with Rtx-1 and Rtx-5ms column. The main compounds of water and ethanolic extract of Turkish propolis were various sugar derivatives, phenolic acids (mainly quinic, ferulic, and caffeic acids), and other compounds.

Discussion

Propolis has recently been used by manufacturers, distributors, and consumers in the world market (especially in medicine, food industry, and cosmetic products) with the increasing popularity in the world and to promote health.^[13,14] For this reason, it is important that the propolis presented for use is of high quality. Unfortunately, the quality control system for propolis and propolis-based products is still not available today.^[13]

The composition of propolis varies widely according to climate, season, location, and year, and therefore its

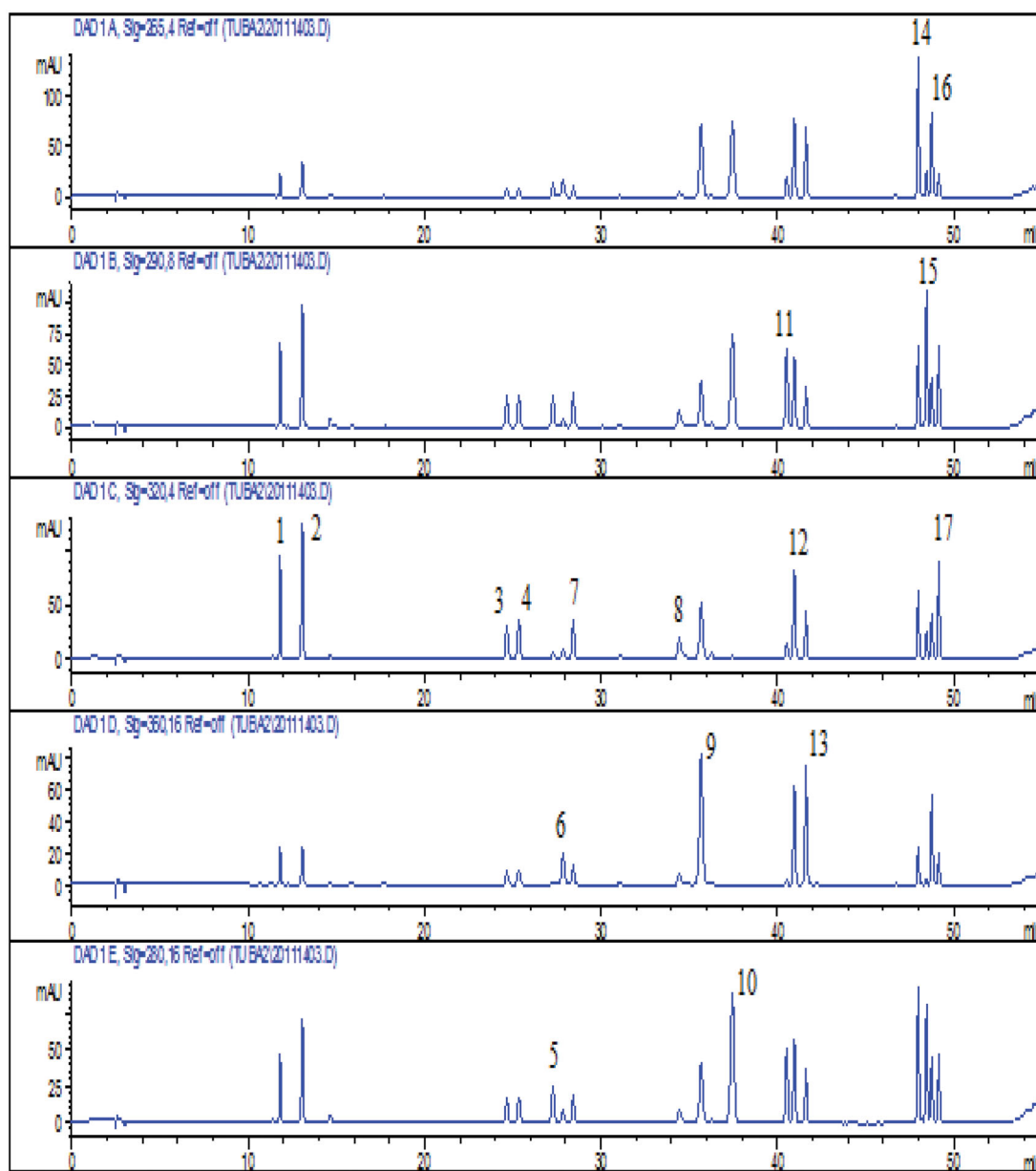


Figure 1. HPLC-DAD chromatograms of standard solution at a wavelength of 265, 290, 320, 360, 280 nm, respectively. (1) Chlorogenic acid; (2) Caffeic acid; (3) 4,5-di-O-caffeoylquinic acid; (4) 3,5-di-O-caffeoylquinic acid; (5) Naringin; (6) Myricetin; (7) 3,4-di-O-caffeoylquinic acid; (8) 3,4,5-tri-O-caffeoylquinic acid; (9) Quercetin; (10) trans-cinnamic acid; (11) Naringenin; (12) Apigenin; (13) Kaempferol; (14) Chrysin; (15) Pinocembrin; (16) Galangin; (17) Caffeic acid phenethyl ester.

Table 2. HPLC-DAD analysis of water and ethanolic extracts of Turkish propolis.

Number	Compounds	Wavelength (nm)	RT (min) (Water extract of Turkish propolis)	Concentration(μ g/mL) (Water extract of Turkish propolis)	RT (min) (Ethanolic extract of Turkish propolis)	Concentration (μ g/mL) (Ethanolic extract of Turkish propolis)
1	Chlorogenic acid	320	12.484	10.20		
2	Caffeic acid	320	13.954	204.00	13.873	66.00
6	Myricetin	360			31.562	2.32
8	3,4,5-tri-O-caffeoylquinic acid	320	39.694	7.75		
9	Quercetin	360			40.132	40.69
10	Trans-cinnamic acid	280			41.136	91.49
11	Naringenin	290	41.309	28.90	43.766	372.39
13	Kaempferol	360			44.491	98.72
14	Chrysin	265			50.737	641.33
15	Pinocembrin	290			51.208	572.67
16	Galangin	265			51.509	534.11
17	Caffeic acid phenethyl ester	320			51.896	630.67

Table 3. GC-MS analysis of water extract of Turkish propolis using Rtx-1 column and their percentages of area.

Compounds	Retention time (RT)	Peak area%
N-Ethyl,N-vinylacetamide	14.098	1.57
N-(trimethylsilyl)-L-Norvaline	14.228	6.50
N,N-diethyl-1,1,1-trimethylsilylamine	14.861	0.33
N,N-diethyl-Acetamide	16.109	2.00
N-ethyl-Acetamide	16.782	1.13
Benzoic acid	26.465	4.39
2,2,8,8-tetramethyl-5-[(trimethylsilyl)oxy]-3,7-Dioxa-2,8-disilanonane	27.845	4.10
Butanedioic acid	29.268	4.06
Hydroquinone	32.818	1.15
[(trimethylsilyl)oxy]-Butanedioic acid	36.416	1.78
D-Psicofuranose (isomer 1)	43.720	2.42
β -D-(-)-Tagatopyranose	43.808	3.99
α -D-(+)-Talopyranose	47.686	4.94
D-(-)-Fructofuranose (isomer 1)	47.898	4.12
D-(-)-Fructofuranose (isomer 2)	48.154	3.14
1,3,4,5,6-pentakis-O-(trimethylsilyl)-D-Fructose	48.345	5.77
6-methoxy-5-(phenylmethoxy)-1H-Indole	48.562	0.79
1,2,3,4,6-pentakis-O-(trimethylsilyl)- β -D-Galactopyranose	50.696	6.39
1,2,3,4,6-pentakis-O-(trimethylsilyl)- β -D-Glucopyranose	53.483	8.98
4-methoxy-3-(trimethylsiloxy)-Cinnamic acid	55.592	1.92
Trimethylsilyl 3,4-bis(trimethylsiloxy)cinnamate	57.372	7.77

Table 4. GC-MS analysis of water extract of Turkish propolis using Rtx-5ms column and their percentages of area.

Compounds	Retention time (RT)	Peak area%
Hydrated formaldehyde	11.619	0.32
N-(trimethylsilyl)acetamide	12.441	0.72
Ethylbis(trimethylsilyl)amine	13.876	8.65
N-ethyl,N-vinylacetamide	14.041	0.93
N-(trimethylsilyl)-L-Norvaline	14.169	4.32
Triisopropylsilane	14.347	0.17
N,N-diethyl-1,1,1-trimethylsilylamine	14.815	0.76
N,N-diethyl-Acetamide	16.078	1.20
2-[(trimethylsilyl)oxy]-Propanoic acid	18.509	0.16
[(trimethylsilyl)oxy]-Acetic acid	19.137	0.13
trimethyl(phenylmethoxy)-Silane	22.522	0.20
trimethyl(2-phenylethoxy)-Silane	25.625	0.14
Benzoic acid	26.458	5.44
2,2,8,8-tetramethyl-5-[(trimethylsilyl)oxy]-3,7-Dioxa-2,8-disilanonane	27.840	4.64
Proline	28.704	0.16
Butanedioic acid	29.262	5.15
Trimethylsilyl 2-acetoxyacetate	29.509	0.31
Hydroquinone	32.824	1.25
Benzenepropanoic acid	33.377	0.20
O-(trimethylsilyl)-Malic acid	36.424	2.64
3-phenyl-2-Propenoic acid	38.212	0.25
2,3,4,5,6-pentakis-O-(trimethylsilyl)-Gulose	47.392	0.23
methyl 2,3,5,6-tetrakis-O-(trimethylsilyl)- α -D-Glucofuranoside	47.576	0.50
D-(-)-Fructofuranose (isomer 1)	47.924	6.24
D-(-)-Fructofuranose (isomer 2)	48.179	4.56
D-(-)-Fructopyranose (isomer 1)	48.371	7.48
1,2,3,4,6-pentakis-O-(trimethylsilyl)-D-Glucopyranose	48.984	0.27
methyl 2,3,5,6-tetrakis-O-(trimethylsilyl)- α -D-Glucofuranoside	49.142	1.52
Quinic acid	49.714	0.23
D-(+)-Galactopyranose (isomer 1)	50.723	6.76
1,2,3,4,6-pentakis-O-(trimethylsilyl)-D-Glucopyranose	51.063	0.36
1,2,3,4,6-pentakis-O-(trimethylsilyl)- α -D-Mannopyranose	53.514	8.57
3,4-dimethoxy-Cinnamic acid	54.153	0.47
4-methoxy-3-(trimethylsiloxy)-Cinnamic acid	55.621	1.70
Ferulic acid	56.075	0.27
Trimethylsilyl 3,4-bis(trimethylsiloxy)cinnamate	57.404	9.54
N-ethyl-Acetamide	61.553	2.59

chemical formula is not stable.^[15] For this reason, chemical analysis of propolis is always needed.^[16] Comparative studies have shown that, although different propolis types have different chemical compositions, these types always have very high biological activities.^[17,18]

Turkey is located between Asia and Europe, surrounded by the Black Sea, the Marmara Sea, the Aegean Sea, and the Mediterranean Sea with highly different climate zones.^[19] Therefore, a wide variety is expected among Turkish propolis samples.^[20] Due to the diversity of vegetation including

Table 5. GC-MS analysis of ethanolic extract of Turkish propolis using Rtx-1 column and their percentages of area.

Compounds	Retention time (RT)	Peak area%
N-ethyl,N-vinylacetamide	14.108	5.61
N-(trimethylsilyl)-L-Norvaline	14.242	28.94
N,N-diethyl-1,1,1-trimethylsilylamine	14.880	5.86
1,2-Bis(trimethylsiloxy)ethane	15.283	2.23
N,N-diethyl-Acetamide	16.105	8.76
2-methyl-(E)-2-Butenoic acid	16.365	0.78
N-ethyl-Acetamide	16.792	4.50
Benzoic acid	26.470	7.64
2,2,8,8-tetramethyl-5-[(trimethylsilyl)oxy]-3,7-Dioxa-2,8-disilanonane	27.847	2.24
Butanedioic acid	29.270	2.31
Hydroquinone	32.825	0.61
3-phenyl-2-Propenoic acid	38.209	0.66
D-(-)-Tagatofuranose (isomer 1)	47.901	0.45
p-methoxy-Cinnamic acid	48.025	0.41
D-Psicofuranose (isomer 1)	48.158	1.65
D-Psicopyranose (isomer 2)	48.342	8.09
D-(+)-Galactopyranose (isomer 1)	50.699	2.02
6-methoxy-5-(phenylmethoxy)-1H-Indole	51.463	1.13
α -D-(+)-Talopyranose	53.485	3.09
3,4-dimethoxy-Cinnamic acid	54.112	1.02
4-methoxy-3-(trimethylsiloxy)-Cinnamic acid	55.591	1.74
Caffeic acid	57.366	1.91
trans-9-Oktadecenoic acid	59.262	0.83

Table 6. GC-MS analysis of ethanolic extract of Turkish propolis using Rtx-5ms column and their percentages of area.

Compounds	Retention time (RT)	Peak area%
Hydrated formaldehyde	11.608	1.56
N-(trimethylsilyl)acetamide	12.473	1.31
N-(trimethylsilyl)-Etanimidic acid	13.497	5.10
Ethylbis(trimethylsilyl)amine	13.888	33.54
N-Ethyl,N-vinylacetamide	14.065	3.68
N-(trimethylsilyl)-L-Norvaline	14.181	18.12
N,N-Diethyl-1,1,1-trimethylsilylamine	14.827	3.46
1,2-Bis(trimethylsiloxy)ethane	15.243	0.91
N,N-diethyl-Acetamide	16.136	4.05
N-ethyl-Acetamide	16.777	2.07
Benzoic acid	26.476	2.39
2,2,8,8-tetramethyl-5-[(trimethylsilyl)oxy]-3,7-Dioxa-2,8-disilanonane	27.850	0.44
Butanedioic acid	29.278	0.77
D-(-)-Fructopyranose (isomer 1)	48.369	1.34

numerous endemic species in Turkey, Turkish propolis samples are different from typical propolis samples in Central and Eastern Europe.^[19,20]

In light of this information, instead of determining the contents of propolis from each region, we aimed to achieve a natural product with much higher biological activity through a synergistic effect of all components of propolis by obtaining a single sample representing each region of Turkey. Thus, we prepared a single propolis sample and obtained a product representing all regions and called it Turkish propolis.

Different studies have found that flavonoids and phenolic compounds are the main components of propolis.^[21–23] In general, these compounds, which are found in the composition of Turkish poplar type propolis, have been reported to show antioxidant activity by some authors.^[24,25]

Since propolis cannot be used and consumed in its raw form, various extraction methods are used to extract the biologically active components.^[7,26] The composition of propolis depends on the extraction method besides its geographical source. Therefore, the solvent to be used in the extraction process should be carefully selected.^[2]

Propolis has low solubility in water and studies are few numbers in such derivatives.^[27] Water extracts exhibit a simpler component profile.^[11] Propolis samples are extracted with water to isolate charged and relatively polar components as cinnamic acid and its derivatives, caffeoyl-quinic acid derivatives, phenolic acids, and esters such as caffeic acid.^[28] Extraction with ethanol is particularly suitable for obtaining a propolis extract rich in resin-free polyphenolic components.^[7]

Silva et al.^[29] by comparing the hydroalcoholic, methanolic, and water extracts of propolis in a study they have concluded that the water and the ethanolic extract are the best solvents for polyphenols. Besides, in a study that we have presented^[30] previously, water, ethanolic, dimethylsulfoxide (DMSO), glycerol and acetone extracts of Turkish propolis collected from the same regions in the present study, total polyphenol and flavonoid content, ferric (Fe^{3+}) reducing power and total antioxidant status were determined. Each extract was also qualitatively analyzed by HPLC. HPLC analysis showed similarities between peaks of DMSO and acetone extracts and peaks of ethanolic and glycerol extracts. As a result of these studies, it was concluded

that propolis was best dissolved in DMSO, then ethanol, acetone, glycerol, and water respectively. Therefore, we preferred to use water and ethanolic extracts of Turkish propolis in our study, because the water extract is nontoxic and may be more suitable for human use, as well as being more soluble and more components are present in the ethanolic extract.

High-performance liquid chromatography coupled to different detectors as a UV-visible detector, a diode-array detector (HPLC-UV, HPLC-DAD), and gas chromatography coupled to mass spectrometry (GC-MS) are widely used in detect and analysis of flavonoids and phenolic compounds present in propolis.^[31,32] Among these, HPLC is undoubtedly the most valid and reliable analytical technique.^[31] Specific detection of polyphenols in propolis is carried out in the UV-Vis region together with DAD. The DAD collects the entire UV spectrum several times during the elution of the chromatographic peak and ensures that all wavelengths of the spectrum are detected simultaneously.^[33]

Different investigators analyzed the polyphenolic compounds of propolis collected from different regions using various extraction systems and various HPLC methods associated with different detector systems.^[10] However, among these studies, no study fully demonstrates the chemical profile, especially water and ethanolic extracts of Turkish propolis.

As a result of our literature research, 17 standards which were determined both in qualitative and quantitative terms for HPLC-DAD analysis were selected because of their higher and more effective ratio in different propolis species prepared with various solvents.^[21,34,35]

Actually, when we look at the literature selecting the same parameters and determining a standardization would be appropriate for the most accurate comparison. However, such an approach seems unlikely since each researcher or research group uses different methods.

Mishima et al.^[36] in a study in which they analyzed the basic components of the water and ethanolic extract of Brazilian propolis by HPLC, found greater amounts of chlorogenic acid, p-coumaric acid, 4,5-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 3,4-di-O-caffeoylquinic acid in the water extract than the ethanolic extract. However, they did not reflect the chromatograms of these compounds in their published article. In our HPLC-DAD analysis, chlorogenic acid and 3,4,5-tri-O-caffeoylquinic acid, which are caffeoylquinic acid derivatives, were found in the water extract of Turkish propolis. It was also found that caffeic acid was higher in water extract compared to ethanolic extract, but trans-cinnamic acid was higher in ethanolic extract. Polyphenolic compounds were found more in the ethanolic extract of Turkish propolis and none of the caffeoylquinic acid derivatives were found.

The amount of caffeic acid in the ethanolic extract of Turkish propolis is approximately 3-fold higher than the amount found by Cardoso et al.^[35] in the ethanolic extract of Brazilian wild green propolis with HPLC-DAD reversed-phase system. However, while the ethanolic extract of Turkish propolis did not contain dicaffeoylquinic acids, 3,4-

dicaffeoylquinic acid, and 3,5-dicaffeoylquinic acid were found in the ethanolic extract of Brazilian wild green propolis. Contrary to this 4,5-dicaffeoylquinic acid was not determined.

In a study analyzed by RP-HPLC, Park et al.^[21] determined kaempferol, pinocembrin, chrysin, and galangin compounds similar to Turkish propolis, in the ethanolic extract of Brazilian propolis and plant resins. In the analysis of ethanolic extract of Brazilian propolis and plant resins by GC-MS, many aromatic compounds, terpenoid, and fatty acid esters have been identified.

Until now various researchers and research groups prepared Turkish propolis samples collected from some cities in Turkey such as Bursa, Muğla, İzmir, Balıkesir, İstanbul, Erzurum, Gümüşhane, Trabzon, Yozgat, Kayseri, Adana, Artvin, Bartın, Ankara, Denizli, Aydın, Konya, Tekirdağ, Rize, Erzincan, Mersin, Yalova, Hatay, Kırklareli, and Çanakkale using 95, 96, or 70% ethanol extracts through different methods and analyzed via GC-MS.^[15,37–45] Apart from these studies, there is no other study that describes the chemical characteristics of Turkish propolis by GC-MS using pure ethanol. Also, there are no studies performed with aqueous extract, except Yildirim et al.^[46] that they prepared the aqueous extract of Turkish propolis obtained from Malatya and determine it with GC-MS.

To determine the content of the water and ethanolic extract of Turkish propolis and to evaluate the difference between the columns, two separate columns with the same branded fillers, Rtx-1 and Rtx-5ms, were used. At the end of our GC-MS analysis, more components were detected in the water extract of propolis with Rtx-5ms column. As a result of these analyses, it was determined that the water and ethanolic propolis extract detected by both columns had much more rich content in terms of various aldoses and carbohydrates than polyphenols. Some important phenolic acids such as quinic acid, ferulic acid, cinnamic acid derivatives were determined in the analyses performed with Rtx-1 and Rtx-5ms columns.

In terms of caffeoylquinic acids and polyphenols in water extract of Turkish propolis, caffeic acid, trans-cinnamic acid, chlorogenic acid, and 3,4,5-tri-O-caffeoylquinic acid were found in HPLC-DAD analysis, while these components were not found in GC-MS analysis. However, quinic acid and cinnamic acid derivatives that form 3,4,5-tri-O-caffeoylquinic acid were determined. In the ethanolic extract of Turkish propolis, chrysin, caffeic acid phenethyl ester, pinocembrin, galangin, naringenin, kaempferol, trans-cinnamic acid, caffeic acid, quercetin, and myricetin were found in HPLC-DAD analysis, while cinnamic acid derivatives were determined in the analysis performed by GC-MS.

Therefore, when we compare HPLC-DAD and GC-MS methods; for the analysis of phytochemicals, GC-MS can be considered as a much more practical, faster, and alternative method than HPLC-DAD. However, HPLC-DAD provides both qualitative and quantitative determination of the caffeoylquinic acids, polyphenols, and flavonoids, which are responsible for the biological activity of propolis, while at the same time it has lower volatility and higher polarity due

to hydroxyl groups. For this reason, HPLC-DAD has emerged as a much more effective, sensitive, reliable, and verified analytical method. GC-MS, on the other hand, was more effective in identifying non-flavonoid compounds. Also, although the analysis time is shorter in GC-MS compared to HPLC-DAD, absolute separation can be made by adjusting the carrier phase with HPLC-DAD and applied to all analytes. Therefore, HPLC-DAD can be considered as a more preferred method for analysis of flavonoids and chemical characterization of propolis than GC-MS.

Conclusion

In conclusion, this study is the first to describe the chemical characterization of water and ethanolic extract of Turkish propolis by HPLC-DAD and GC-MS analysis. HPLC-DAD analyses of Turkish propolis revealed that high concentrations of caffeic acid were present in the water extract of Turkish propolis; chrysin, caffeic acid phenethyl ester, pinocembrin, galangin, and naringenin were present in the ethanolic extract of Turkish propolis. Moreover, GC-MS analyses of Turkish propolis revealed that caffeic, ferulic, and quinic acids besides benzoic acid and sugar derivatives were present in the propolis.

All these chemical analyses showed that the water and ethanolic extracts of Turkish propolis contain high amounts of flavonoid, phenolic acid, and caffeoylquinic acid, which have versatile biological activities, and the majority of these samples exhibit similar phenolic profile. However, the quantitative differences in the studies of the literature are due to the concentration of propolis extract, the type of solvent used, and the wavelength chosen. Also, HPLC-DAD can be considered as a more effective method than GC-MS for the chemical characterization of Turkish propolis.

Although the number of soluble compounds in the ethanol extract is much higher and contains a high amount of phenolic components, the water extract is nontoxic, can be prepared without any extra cost and effort, and it is a natural product that is extremely suitable for human use thanks to its high antioxidant content. Turkish propolis can be a good source of raw materials for sectors such as the food and pharmaceutical industry.

In addition, this study can provide an idea about which of the water and ethanolic extracts is used for various studies to be carried out on biological activity in the future.


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ORCID

Tuğba Nigar Bozkuş  <http://orcid.org/0000-0001-9613-6911>
Orhan Değer  <http://orcid.org/0000-0003-3584-6324>
Ahmet Yaşar  <http://orcid.org/0000-0002-5487-1536>

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